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Preventive and curative activity of combined treatments of sodium carbonates and *Pantoea agglomerans* CPA-2 to control postharvest green mold of citrus fruit

J. Usall[a,*], J. Smilanick[b], L. Palou[c], N. Denis-Arrue[b], N. Teixidó[a], R. Torres[a], I. Viñas[d]

[a] *IRTA, Centre UdL-IRTA, XaRTA-Postharvest, Av. Rovira Roure 191, 25198 Lleida, Catalonia, Spain*
[b] *San Joaquin Valley Agricultural Sciences Center, USDA-ARS, 9611 South Riverbend Avenue, Parlier, CA 93648, USA*
[c] *Centre de Tecnologia Postcollita, Institut Valencià d'Investigacions Agràries (IVIA), Apartat Oficial, 46113 Montcada, València, Spain*
[d] *UdL, Centre UdL-IRTA, XaRTA-Postharvest, Av. Rovira Roure 191, 25198 Lleida, Catalonia, Spain*

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ABSTRACT

Preventive and curative activity of 2 min dips in 3% sodium carbonate (SC) or sodium bicarbonate (SBC) aqueous solutions heated to 40 °C, alone or followed by the application of 2×10^8 CFU/mL of the biocontrol agent *Pantoea agglomerans* CPA-2 (BA), were simultaneously evaluated for the control of postharvest green mold, caused by *Penicillium digitatum*, in artificially inoculated Lanelate and Valencia oranges. Fresh cells of BA proliferated inside rind wounds and their survival was not adversely affected by the presence of residues of SC or SBC. Green mold incidence after 7 d of incubation at 20 °C in rind wounds treated after fungal inoculation (curative activity) was 15%, 40%, or 15% in oranges treated with SC, BA, or SC + BA and 5%, 45% or 0% in oranges treated with SBC, BA, or SBC + BA, respectively, while it was about 90% in untreated control fruit. Green mold incidence in rind wounds treated before inoculation or reinoculation with the pathogen (preventive activity in pre-existing wounds) was 10% and 2%, or 15% and 8%, respectively, in oranges treated with SC and SC + BA, and 3% and 5%, or 20% and 5%, respectively, in oranges treated with SBC and SBC + BA. Green mold incidence in wounds inoculated after treatment (preventive activity in new wounds) was 55% and 25%, and 60% and 40% in oranges treated with SC and SC + BA, or SBC and SBC + BA, respectively. Additionally, the duration of the protective effect of SBC, BA, and SBC + BA was assessed in Eureka lemons and Valencia oranges. In both species, all three treatments effectively protected pre-existing rind wounds during 7 d of storage at 10 °C. After 0, 1, and 2 d, but not after 4 or 7 d, the protective effect of SBC was significantly inferior to that of BA and SBC + BA. The integration of treatments is a promising approach to replace the use of conventional fungicides to control green mold in citrus packinghouses.

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1. Introduction

Green mold, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is the most economically important postharvest disease of citrus in Spain, California, and all production areas characterized by low summer rainfall (Eckert and Eaks, 1989). *P. digitatum* is a strict wound pathogen that can infect the fruit in the grove, the packinghouse, and during distribution and marketing. Its conidia are ubiquitous within groves and packinghouses and easily transported by air currents from rotting to sound fruit. When the frequency of fungicide-resistant isolates is low, decay is adequately controlled by synthetic fungicides. Fungicides such as imazalil or thiabendazole have curative action, controlling pre-existing infections, and deposit a fruit residue that prevents subsequent infections and retards sporulation from those few that do develop (Ismail and Zhang, 2004). However, their widespread use in packinghouses has led to the proliferation of fungicide-resistant isolates in California (Kinay et al., 2007) and Spain (Díaz and Vila, 1989; Palou et al., 2001b). Furthermore, alternatives to conventional fungicides are needed because of concerns about environmental contamination and human health risks associated with their residues.

Biological control using microbial antagonists is a promising alternative to fungicides to control postharvest diseases of citrus. Studies at our laboratory in the UdL-IRTA Centre have shown that the strain CPA-2 of the bacterium *Pantoea agglomerans*, isolated from an apple surface, is an effective antagonist to the major postharvest pathogens of citrus and pome fruit (Usall et al., 2001; Teixidó et al., 2001; Nunes et al., 2002). However, these and other

* Corresponding author.
E-mail address: josep.usall@irta.cat (J. Usall).

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biocontrol agents cannot provide by themselves the consistent or broad-spectrum control of synthetic fungicides. In general, microbial antagonists show great variability in their efficacy, confer only a protective effect that diminishes with ripening, usually cannot eradicate incipient or pre-existing infections, and do not prevent fungal sporulation (El Ghaouth et al., 2002). Therefore, considerable effort has been devoted recently to develop a 'next generation' of biological control products (Droby et al., 2003). Janisiewicz and Korsten (2002) stated the primary approaches to improve biocontrol of postharvest diseases are manipulation of the environment, use of mixtures of antagonists, physiological and genetic manipulation of antagonists, combining field and postharvest applications, manipulation of formulations, and integration with other non-biological methods.

Carbonic acid salts such as sodium carbonate (SC, Na_2CO_3, soda ash) or sodium bicarbonate (SBC, $NaHCO_3$), are good candidates to be used in combination with other chemical, physical, or biological methods for the integrated control of postharvest citrus diseases (Smilanick et al., 2005, 2006; Palou et al., 2007). They are common food additives allowed with no restrictions for many applications by European and North American regulations. Both salts can be useful tools to manage citrus postharvest decay because in addition to their considerable antimicrobial activity, they are inexpensive, readily available, and can be used with a minimal risk of injury to the fruit. When applied alone, sodium carbonates are effective in controlling green mold on lemons (Smilanick et al., 1995), oranges (Smilanick et al., 1997, 1999; Palou et al., 2001a) and, with less effectiveness, clementine mandarins (Palou et al., 2002a). Although, it has not been completely elucidated, their mode of action appears to be primarily fungistatic and, in contrast to the synthetic fungicides mentioned previously, these salts are not very persistent fungicides. Biocontrol antagonists, conversely, persist for long periods after application and, although they are poor eradicants that are generally incapable of controlling established infections, they could provide persistent protection of the fruit from possible reinfection during postharvest handling and storage. Combinations of SBC and *P. agglomerans* CPA-2 were superior to either treatment alone to control green mold on oranges and clementine mandarins (Teixidó et al., 2001; Torres et al., 2007). Moreover, similar results occurred when SBC or SC were combined with other biocontrol antagonistic such as *Pseudomonas syringae* (Smilanick et al., 1999), *Candida oleophila* (Porat et al., 2002; Lanza et al., 2004), or *Bacillus subtilis* (Obagwu and Korsten, 2003). These studies primarily described control of pre-existing infections of *P. digitatum* (curative activity) and the fruit were artificially inoculated with the pathogen at different times before the application of the treatments. In some cases, the ability of combined treatments to protect the fruit from future infections was evaluated (preventive activity) and the fruit were inoculated with the pathogen after treatment. When both curative and preventative activity were evaluated by these authors, it was typically done by conducting separate tests with different fruit for each type of evaluation.

In the present work, we simulated commercial situations in which reinfection of the same fruit may occur during handling and processing within the packinghouse. To do this, we re-inoculated with *P. digitatum* (at either the same or different rind infection sites) fruit that had been previously inoculated or just wounded, and treated with sodium carbonates, the biocontrol agent *P. agglomerans* CPA-2, or the combination of these treatments. Different sequences of treatment and fungal re-inoculation were tested in order to evaluate the curative and protective activity of single and combined treatments and to establish the optimum point in the packinghouse fruit handling sequence for treatment application. Furthermore, the duration of the protective effect of these treatments was assessed by inoculating the fungus at different times after storage in previously wounded-only and treated fruit. Additionally, the population dynamics of *P. agglomerans* CPA-2 in fresh wounds or wounds treated with sodium carbonate or sodium bicarbonate were also determined.

2. Materials and methods

2.1. Fruit

Oranges (*Citrus sinensis* (L.) Osbeck) cvs. Lanelate or Valencia, from commercial orchards in the San Joaquin Valley (California) or southern Tarragona (Catalonia), and lemons (*Citrus limon* (L.) Burm.) cv. Eureka, from commercial orchards in California, were selected from field bins after harvest and used in the experiments before any commercial postharvest treatments were applied. The fruit were used the same day, or stored up to 2 weeks at 5 °C and 90% relative humidity (RH) before use.

2.2. Fungal inoculum

Petri dishes of potato dextrose agar (PDA) were inoculated with *P. digitatum* isolate PIM-1 and incubated at 25 °C for 7–10 d. Conidia were rubbed from the agar surface with a sterile glass rod after 5 mL of 0.05% (w/v) Triton X-100 in water was added. The conidial suspension was passed through two layers of cheesecloth and diluted with water to an absorbance of 0.1 at 420 nm determined with a spectrophotometer. This density is approximately equivalent to 1×10^6 spores/mL (Morris and Nicholls, 1978) and this inoculum density is recommended for evaluation of postharvest treatments to control citrus green mold (Eckert and Brown, 1986).

2.3. Biocontrol agent

P. agglomerans strain CPA-2 was obtained from the UdL-IRTA Centre (Catalonia). It was originally isolated from the surface of an apple (cv. Golden Delicious). Bacterial suspensions for efficacy and population assays were prepared by growing cultures in flasks half-filled with a medium containing yeast extract (5 g/L) and sucrose (10 g/L) for 24 h at 30 °C and 150 rpm. The medium was centrifuged at 10,000 × *g* for 11 min at 10 °C (RC5C Sovall Instruments Dupont, Newton, CT) and the cell paste was resuspended in 0.05 M phosphate buffer (pH 7.2) to the desired concentration of 2×10^8 colony-forming units per millilitres (CFU/mL).

2.4. Population dynamics of *P. agglomerans* CPA-2 in fresh or treated wounds

Valencia oranges from Tarragona were wounded once on the equator with a stainless steel rod with a probe tip 1 mm wide and 2 mm in length, placed in metallic grid baskets, and immersed for 2 min in fresh water (control), 3% (w/v) SC (pH 11.3–11.5; Sigma–Aldrich, St. Louis, MO, USA), or 3% SBC (pH 8.3–8.6; Sigma–Aldrich) solutions at room temperature (20 ± 2 °C). Salt-treated fruit were not rinsed. Treated fruit were placed into plastic cavity trays, allowed to air-dry at room temperature, then inoculated in the rind wound with 15 μL of *P. agglomerans* at 2×10^8 CFU/mL, and incubated at 20 °C for 7 d. Bacterial populations were determined after 0, 1, 4, and 7 d of incubation. A circular 2.5 cm^2 piece of peel that included the inoculated wound was removed with a sterile cork borer from each orange. Peel surface was homogenized in 50 mL of 0.05 M phosphate buffer. Serial 10-fold dilutions of the washings were made and plated on nutrient yeast dextrose agar (NYDA) medium. Colonies were counted after

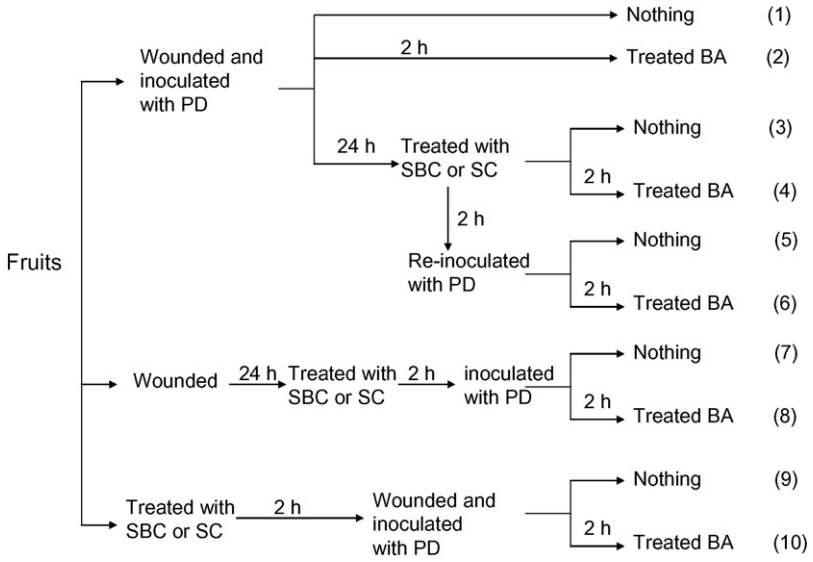

Fig. 1. Flow chart of treatments.

incubation at 25 °C for 48 h. One fruit per treatment and four replicates per treatment were used. Population sizes were expressed as CFU/wound.

2.5. Treatments protocols

The following 10 treatments were applied on Lanelate and Valencia oranges from California (Fig. 1): (1) fruit were wound inoculated with *P. digitatum* (control treatment); (2) fruit were wound inoculated with *P. digitatum* then treated 2 h later in the same wound with the strain CPA-2 of the biocontrol agent *P. agglomerans* (BA); (3) fruit were wound inoculated with *P. digitatum* and treated 24 h later with sodium carbonate (SC) or sodium bicarbonate (SBC); (4) fruit were wound inoculated with *P. digitatum*, treated 24 h later with SC or SBC, and treated 2 h later in the same wound with *P. agglomerans*; (5) fruit were wound inoculated with *P. digitatum*, treated 24 h later with SC or SBC, and reinoculated 2 h later in the same wound with *P. digitatum*; (6) fruit were wound inoculated with *P. digitatum*, treated 24 h later with SC or SBC, reinoculated 2 h later in the same wound with *P. digitatum*, and treated 2 h later in the same wound with *P. agglomerans*; (7) fruit were wounded, treated 24 h later with SC or SBC, and inoculated 2 h later in that wound with *P. digitatum*; (8) fruit were wounded, treated 24 h later with SC or SBC, inoculated 2 h later in that wound with *P. digitatum*, and treated 2 h later in the same wound with *P. agglomerans*; (9) fruit were treated with SC or SBC, and wounded and inoculated 2 h later with *P. digitatum*; and (10) fruit were treated with SC or SBC, wounded and inoculated 2 h later with *P. digitatum* and then treated 2 h later in this wound with *P. agglomerans*.

The first fungal inoculation was performed by immersing a stainless steel rod with a probe tip 1 mm wide and 2 mm in length into the 1×10^6 spores/mL suspension of *P. digitatum* and wounding each fruit once on the equator. Wounds on fruit that were initially wounded but not inoculated were inflicted with the same rod without immersing it in the inoculum suspension.

SC and SBC treatments were done by placing the oranges into plastic baskets and immersing them for 2 min in 22-L tanks containing 3% (w/v) SC or SBC solutions at 40 °C. A control treatment of heated water alone at 40 °C was omitted, because a treatment of this duration and water temperature does not retard green mold development (Palou et al., 2001a). The treatment equipment consisted of 12 stainless steel tanks, each individually fitted with a computer-controlled electrical heater, a temperature sensor, and a mechanical agitation system. The temperature of the solutions did not change more than 0.5 °C during treatment. Fruit treated with carbonates were immediately rinsed with 10 mL of deionized water per fruit at low pressure (about 200 kPa) in a spray 30 cm above the fruit for 5 s, placed in plastic cavity trays, and allowed to dry in air at room temperature.

Fungal reinoculations were performed about 2 h later when the fruit were completely dried by placing 10 μL of a 1×10^6 spores/mL suspension of *P. digitatum* in either previously made or new rind wounds. Reinoculations into new wounds were performed to simulate potential wounding and infection during packinghouse handling and processing of fruit already treated with carbonates.

Once the droplet of fungal inoculum had dried, the biocontrol agent was treated by placing 15 μL of a 2×10^8 CFU/mL suspension of *P. agglomerans*.

Treated fruit were incubated at 20 °C for 7 d, at which time the incidence of green mold on both previously made and new wounds was determined. Each treatment was applied to 3 replicates of 10 oranges each. The experiment was repeated three times in California, once with Lanelate oranges and twice with Valencia oranges.

2.6. Assessment of temporal protective activity

The ability of treatments with SBC, BA, and the combination of both treatments to protect the treated fruit from reinoculation by *P. digitatum* during different time periods was assessed on lemons and oranges. The following treatments were applied: (1) fruit were wounded once on the equator with a stainless steel rod (control treatment); (2) fruit were wounded, treated with 3% SBC at 40 °C for 2 min, and rinsed with fresh water (SBC treatment); (3) fruit were wounded and treated in the wound with 15 μL of a 2×10^8 CFU/mL suspension of *P. agglomerans* CPA-2 (BA treatment); and (4) wounded and SBC-treated fruit were allowed to air-dry and then the bacterial antagonist was applied to the same wound (combined treatment, SBC + BA). All these treatments were applied as previously described.

After treatment, fruit were stored at 10 °C for 0, 1, 2, 4, or 7 d. At the end of each one of these time periods, fruit were inoculated in the initial rind wound with 10 μL of a 1×10^6 spores/mL suspension of *P. digitatum*. Fruit inoculated with the fungus were

stored at 10 °C for 14 d, at which time the number of infected fruits was counted. For each combination of treatment and preinoculation storage period, 4 replicates of 10 oranges each were used. The experiment was repeated three times, once with Eureka lemons in California and twice with Valencia oranges in Catalonia.

2.7. Statistical analyses

Analysis of variance was applied to the arcsine of the square root of the proportion of decayed fruit using SAS software (SAS Institute Inc., Cary, NC). When appropriate, means were separated by Fisher's protected LSD test ($P < 0.05$). *P. agglomerans* population on wounded oranges (CFU/wound) were log transformed to improve homogeneity of variances and plotted in figures where standard deviation errors are shown for each sampling date.

3. Results

3.1. Population dynamics of P. agglomerans CPA-2 in fresh or treated wounds

Populations of *P. agglomerans* CPA-2 in wounds in the rind of oranges treated with water (BA) or SBC (SBC + BA) increased more than 10-fold during the first 24 h of incubation at 20 °C to a mean of 6.3×10^6 CFU/wound. Then, the population gradually decreased to about 3×10^6 CFU/wound after 4 d of incubation and remained constant until the end of the 7-d incubation period (Fig. 2). The pattern of growth was similar on oranges treated with SC and inoculated with the biocontrol agent (SC + BA).

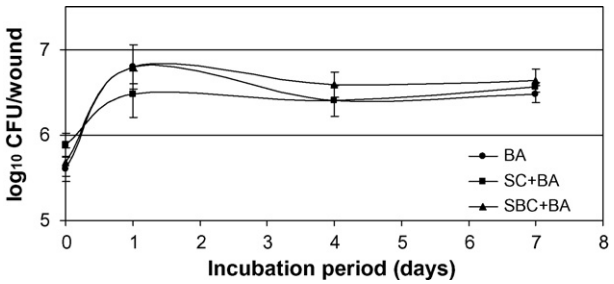

Fig. 2. Population dynamics of *P. agglomerans* CPA-2 applied on wounded Valencia oranges after dipping the fruit in fresh water (BA), 3% (w/v) sodium carbonate (SC + BA), or 3% sodium bicarbonate (SBC + BA). The solutions were applied at room temperature for 2 min. After treatment, the fruit were inoculated in the wound with 15 μL of *P. agglomerans* at 2×10^8 CFU/mL and incubated at 20 °C for 7 d. Values represent the means of four replicates and vertical bars indicate standard deviation of the means.

3.2. Treatments

Treatment of inoculated fruit with SC reduced green mold incidence from 83% among the inoculated and untreated control fruit to 15%, indicating good curative activity (Fig. 3). The curative effect was not enhanced by BA application following SC treatment. When applied alone, BA also showed curative activity (green mold incidence of 40%) but it was significantly inferior to that of SC. On the other hand, SC treatment showed an acceptable protective effect since decay incidence was only 15% on oranges reinoculated with *P. digitatum* in the same wound after SC treatment, and it was just about 8% on oranges treated with BA after reinoculation with the pathogen. The protective effect of SC treatment was also notable on wounded oranges that were inoculated later with the fungus, and the addition of BA to this treatment did not significantly reduce green mold incidence. When oranges were inoculated with the pathogen in a new rind wound after SC treatment, green mold incidence was 56%, indicating that SC treatment only protected the fruit modestly from infections in new wounds. When SC treatment and subsequent fungal inoculation were followed by the application of BA, decay incidence in new wounds was reduced to 27%, showing that the treatment with the antagonist significantly compensated for the lack of protective activity of SC treatment.

Similar results were obtained with treatments of SBC, BA, and their combination (Fig. 4). While green mold incidence was 90% in control fruit and 45% in fruit treated with BA alone, it was 5% and 0% after application of SBC or the combination SBC-BA, respectively. The curative activity of these treatments, therefore, was similar to that of SC or the combination SC-BA. Effectiveness of SBC treatment in protecting inoculated or not inoculated wounds from infection was comparable to that of SC. Similarly, the addition of BA to SBC-treated and reinoculated fruit did not significantly improve green mold control. Like SC, SBC treatment provided poor protection against infections in wounds made and inoculated with the fungus

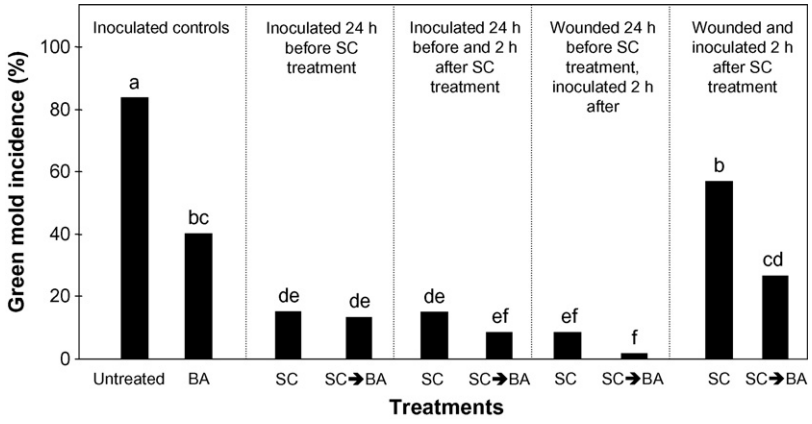

Fig. 3. Influence of time and site of inoculation of oranges with *P. digitatum* on the effectiveness of *P. agglomerans* CPA-2 (BA) and sodium carbonate (SC) treatments applied alone or in sequence (SC → BA) to control green mold. Inoculated controls included untreated fruit and fruit treated with BA 2 h after inoculation. SC was applied by immersion of the fruit in 3% (w/v) SC solution at 40 °C for 2 min followed by a brief water rinse. BA treatment consisted of the application of 15 μL of 2×10^8 CFU/mL of *P. agglomerans* in the inoculated wounds. On fruit inoculated before SC treatment, BA was applied 2 h after SC treatment. On fruit inoculated after SC treatment, *P. digitatum* was inoculated 2 h after SC treatment and BA was applied 2 h after fungal inoculation. After every treatment, the fruit were incubated at 20 °C for 7 d. Values are the means of three experiments, one with Lanelate and two with Valencia oranges. Columns with the same letter are not significantly different according to Fisher's protected LSD test ($P < 0.05$) applied after an analysis of variance to the arcsine-transformed data. Non-transformed data are shown.

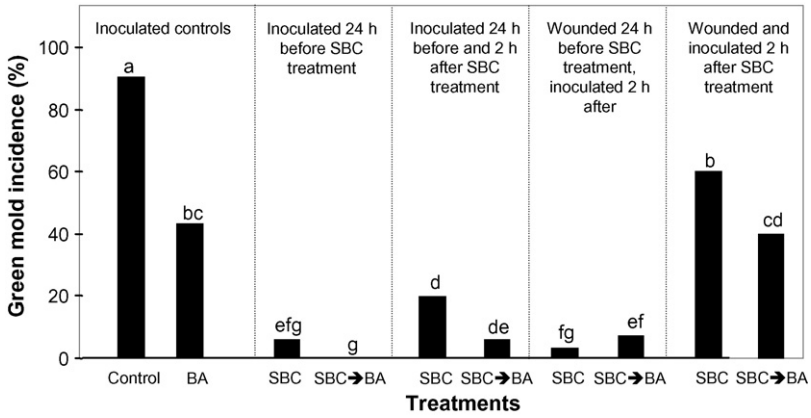

Fig. 4. Influence of time and site of inoculation of oranges with *P. digitatum* on the effectiveness of *P. agglomerans* CPA-2 (BA) and sodium bicarbonate (SBC) treatments applied alone or in sequence (SBC → BA) to control green mold. Inoculated controls included untreated fruit and fruit treated with BA 2 h after inoculation. SBC was applied by immersion of the fruit in 3% (w/v) SBC solution at 40 °C for 2 min followed by a brief water rinse. BA treatment consisted of the application of 15 μL of 2×10^8 CFU/mL of *P. agglomerans* in the inoculated wounds. On fruit inoculated before SBC treatment, BA was applied 2 h after SBC treatment. On fruit inoculated after SBC treatment, *P. digitatum* was inoculated 2 h after SBC treatment and BA was applied 2 h after fungal inoculation. After every treatment, the fruit were incubated at 20 °C for 7 d. Values are the means of three experiments, one with Lanelate and two with Valencia oranges. Columns with the same letter are not significantly different according to Fisher's protected LSD test ($P < 0.05$) applied after an analysis of variance to the arcsine-transformed data. Non-transformed data are shown.

after SBC treatment. As with SC, green mold control in these new wounds was significantly improved when BA treatment followed fungal inoculation.

3.3. Assessment of temporal protective effect

The natural susceptibility to infection by *P. digitatum* of untreated rind wounds in both Eureka lemons (Fig. 5A) and Valencia oranges (Fig. 5B) diminished with time, particularly after 4 d and especially on oranges. More than half of these wounds, however, remained infectable even after 7 d of storage at 10 °C.

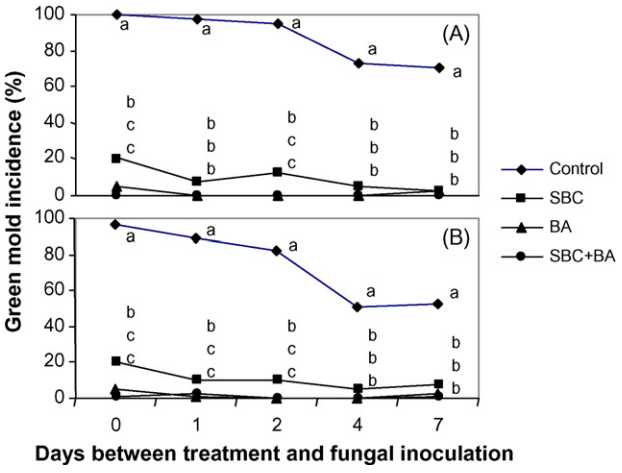

Fig. 5. Green mold incidence on Eureka lemons (A) and Valencia oranges (B) inoculated with *P. digitatum* after 0, 1, 2, 4, or 7 d of storage at 10 °C. Fungal inoculation was performed in artificial rind wounds inflicted to the fruit before storage. Stored fruit had been previously wounded (control), wounded and dipped for 2 min in a 3% (w/v) sodium bicarbonate solution at 40 °C (SBC), wounded and inoculated with the biocontrol agent *P. agglomerans* CPA-2 (BA), or wounded, treated with sodium bicarbonate and then inoculated with *P. agglomerans* CPA-2 (SBC + BA). Green mold incidence was determined after 14 d of storage at 10 °C following fungal inoculation. Values are the means of one and two experiments with lemons and oranges, respectively. For each period between treatment and fungal inoculation, means with the same letter are not significantly different according to Fisher's protected LSD test ($P < 0.05$) applied after an analysis of variance to the arcsine-transformed data. Non-transformed data are shown.

Irrespective of fruit species and period between treatment and inoculation with *P. digitatum*, green mold incidence after 14 d of storage at 10 °C was significantly lower on wounded fruit previously treated with SBC, BA, or the combination SBC + BA than on wounded and untreated fruit (control fruit). Protection from fungal infection in wounds treated with SBC and/or BA persisted for 7 d and decay incidence on previously treated fruit was lower than 20% in all cases. All three treatments, therefore, satisfactorily protected pre-existing rind wounds from *P. digitatum* infections. However, the protective effect of SBC on both lemons and oranges was significantly lower (10–20% of infected fruit) than that of BA or SBC + BA (0–5% of infected fruit) when the period of time between treatment and fungal inoculation was 0, 1, or 2 d. In contrast, this significant difference was not observed when fungal inoculation was elapsed by 4 or 7 d.

4. Discussion

Because of the epidemiological factors, effective control of green mold requires treatments that simultaneously provide both curative and preventive activities. This is the first time, to our knowledge, that both activities were evaluated together in the same fruit. By using different sequences of wounding, inoculation with *P. digitatum*, treatment, and fungal reinoculation in the rind of the same fruit, the ability of the antifungal treatments to control existing infections and/or protect the fruit from subsequent infections was thoroughly characterized.

Teixidó et al. (2001) demonstrated that the strain CPA-2 of *P. agglomerans* was able to effectively colonize wounds on the peel of citrus fruit at either room or low temperature, although it had a limited growth on the surface of the peel. This is an advantageous characteristic because, once applied, the bacterium grows in wound sites where it can interfere with the pathogen to prevent disease and decrease to non-detectable or very low populations on fruit surface. Furthermore, fresh cells of the antagonist were totally tolerant and compatible with a solution of 2% SBC and they were able to proliferate inside wounds in the rind of oranges that had been previously treated with this solution. More recent work by Torres et al. (2007) showed that the formulation FD10-3 of *P. agglomerans* CPA-2, a formulation consisting of freeze-dried cells with 10% sucrose stored at 4 °C for 3 months, also survived in wounds treated with 3% SBC. In this work, we found that the survival of fresh cells of

P. agglomerans inside rind wounds during incubation at 20 °C was not adversely affected by the presence of residues of either SBC or SC that had been applied as aqueous solutions at a concentration of 3%. While this result was expected for SBC, it was surprising for SC because it had been reported that *P. agglomerans* hardly survived after 30 min in contact with 2% SC at 25 °C in glass test tubes (Teixidó et al., 2001). Therefore, the *in vivo* interaction between the cells of the antagonist and the salt residue in a citrus rind wound differs considerably from that which occurs when the cells are suspended in the salt solution. Survival of *P. agglomerans* CPA-2 in wounds is especially important because, in contrast to other biological control agents which modes of action include antibiosis or induction of disease resistance in the fruit host tissues, it has been observed that the antagonistic action of this strain against *P. digitatum* or *P. italicum* is primarily based on the physical contact with the pathogen in the infection site and also on competition for nutrients (Poppe et al., 2003).

In agreement with previous research work, we found that dips in SC or SBC heated aqueous solutions (Smilanick et al., 1999; Palou et al., 2001a, 2002a) and treatments with the biocontrol agent *P. agglomerans* CPA-2 controlled to some extent green mold on citrus fruit that had been previously inoculated with the pathogen (Usall et al., 2001; Viñas et al., 2001) and that the combination of treatments was more effective than either treatment alone (Teixidó et al., 2001; Torres et al., 2007). The mode of action of *P. agglomerans* CPA-2 against *Penicillium* spp. in citrus fruit can explain the limited curative activity of the antagonist when applied alone. The inhibitory effect due to competition for nutrients and space is considerably lower when the bacterium is applied after the pathogen is already established and actively growing within the infection site in the fruit peel. Because of this disadvantage in the competition, *P. agglomerans* CPA-2 and many other antagonistic microbes, mainly yeasts, which mode of action is not antibiosis or parasitism, are poor eradicants of pathogens infecting citrus fruit in the field or in rind wounds inflicted during or just after harvest (Droby et al., 1998; Spadaro and Gullino, 2004). Likewise, the ability to control these incipient infections is usually improved by the integration of these biocontrol treatments with other physical or chemical treatments that, like heated carbonate solutions, provide synergistic or additive inhibitory effects (El Ghaouth et al., 2002; Porat et al., 2002; Obagwu and Korsten, 2003). In our case, treatment of previously inoculated oranges with heated SBC followed by the application of BA was superior to the combination with heated SC and completely controlled green mold. This result corroborated the satisfactory curative action of combined treatments of SBC and *P. agglomerans* against citrus green and blue molds recently reported by Teixidó et al. (2001) and Torres et al. (2007).

In this work, we found, somewhat unexpectedly, that both 3% SC and SBC dip treatments at 40 °C effectively protected pre-existing wounds from fungal infections that took place about 2 h after treatment (green mold incidence lower than 20%; Figs. 3 and 4), and that the protective effect of 3% SBC dips at 40 °C was very persistent because green mold incidence was also lower than 20% in wounds inflicted and treated up to 2 d before inoculation and even lower than 10% in wounds produced and treated 4 or 7 d before inoculation (Fig. 5). This result was not expected because the effects of carbonate salts and other low-toxicity food additives or GRAS compounds against a variety of postharvest pathogens including *P. digitatum* are believed to be more fungistatic than fungicidal and not very persistent (Smilanick et al., 1999; Palou et al., 2001a, 2002b). Survival of spores of *P. italicum* recovered from rind wounds that had been treated with carbonate solutions about 2 h after fungal inoculation (ungerminated spores at the moment of the treatment) was reported by Palou et al. (2001a), Porat et al. (2002) observed that most of the spores of *P. digitatum* recovered in a similar way were actually killed if the fruit were treated with SBC 24 h after fungal inoculation (the spores were thus germinated at the moment of the treatment). In any case, it can reasonably be concluded from these and other studies (Smilanick et al., 1999) that the *in vivo* inhibitory effect of these compounds in infected citrus fruit cannot be explained by their *in vitro* toxicity and it is more likely due to the unfavorable environmental conditions for fungal development that occur within the wound infection courts occupied by the fungus as a consequence of the presence of salt residues. This hypothesis might be confirmed by the tests we report here because while green mold incidence was satisfactorily reduced in old peel wounds that were immersed in the salt solutions soon after being produced, thus containing considerable SC or SBC residues, it was not in new wounds that were made in the peel and inoculated with the pathogen just after the treatment with the salts. The actual amount of salt residues present inside these new wounds was presumably very low, especially because the fruit was briefly rinsed with tap water after being dipped in the salt solutions.

A result we expected was that the preventive or protective activity of the treatments with SC or SBC was usually improved when they were followed by the application of *P. agglomerans* CPA-2. In the experiments where fruit inoculation followed closely the application of the combined treatments, were not statistically differences in preventive activity (Figs. 3 and 4). In the tests where the temporal protective effect of the treatments was assessed in lemons and oranges, when the period of time between treatment and fungal inoculation was 0, 1, or 2 d, green mold incidence was significantly lower after the application of either the antagonist alone or the combined treatment than after the application of SBC alone. In this case, the combination of treatments did not improve the excellent protection provided by the antagonist alone. In contrast, when that period of time was increased to 4 or 7 d, those differences were not significant (Fig. 5). This appeared to be related with the natural susceptibility of the wounds to infection by *P. digitatum*; while green mold incidence on control fruit was about 70% on lemons and 50% on oranges when the time between treatment and fungal inoculation was 4 or 7 d, it was about 100% and 80%, respectively, when that time was 2 d. In spite of this result, it can be concluded that the overall protection of wounds provided by the application of *P. agglomerans* CPA-2 was superior to that provided by the treatment with carbonates. Another original observation in this work was the lasting susceptibility of surface wounds in the peel of lemons and oranges to infection by *P. digitatum* under our experimental conditions. More than half of the wounds that were inflicted in both fruit species remained susceptible even after 7 d of incubation at 10 °C (Fig. 5). This result emphasizes the need to adopt adequate commercial procedures that minimize the risk of damaging the fruit during postharvest handling in citrus packinghouses because, the surface wounds can be infected by the pathogen for several days. Although, the integration of carbonate treatments with *P. agglomerans* CPA-2 consistently provided good protective activity in pre-existing wounds, green mold reduction was lower in new wounds that were inflicted and inoculated with the fungus after the application of the treatments.

Our work shows that a strategy that combines several alternative control methods is promising to effectively control postharvest green mold of citrus fruit and replace conventional fungicides used now in citrus packinghouses. The proposed sequence of treatments would comprise initial treatment with heated solutions of SC or SBC followed by the application of BA. As in other studies (Smilanick et al., 1999; Palou et al., 2001a, 2002a), SC and SBC treatments showed similar effectiveness against green mold, but disposal issues related to the pH and the amount of sodium in residual solutions may make the commercial use of SBC more advisable than the use of SC. These results should be validated through large-scale trials to demon-

strate the value of these treatments to the citrus industry. Nevertheless, the good commercial performance of similar sequences of alternative treatments, namely the integration of short SBC treatments at high temperature (50 °C) with a biocontrol formulation based also on the antagonistic bacterium *P. agglomerans* CPA-2, has been proved in semi-commercial and commercial trials conducted with oranges and mandarins from different Mediterranean locations (Torres et al., 2007). In our opinion, such combined treatments are effective and reliable enough to be implemented at a commercial scale in many citrus packinghouses because they are compatible with existing facilities and postharvest handling practices and would imply minimal environmental and worker safety concerns. Currently, the main handicap to the adoption of such antifungal treatments by the citrus industry in Europe is the strict regulatory issues that exist regarding the registration of biological control commercial products, especially if compared to less restrictive policies of other countries such as the USA (Alabouvette et al., 2006).

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